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Gametophyte Development and Antheridiogen Activity in *Thelypteris ovata* var. *lindheimeri*

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ABSTRACT.—Gametophyte development was studied in *Thelypteris ovata* var. *lindheimeri* from spore germination to 48 days. Spores were sown aseptically onto the surface of agar-solidified mineral medium in multispore cultures. Germination began four days after spore sowing. Gametophytes were spatulate in morphology at six days and terminal meristems were initiated at ten days. Antheridia were observed at 19 days, archegonia at 21 days. At 28 days, gametophytes were antheridiate (smaller, 0.6–1.0 mm in width), archegoniate (larger, 1.3–2.3 mm in width) or asexual (variable, 0.5–1.5 mm in width). Gametophytes remained unisexual at 48 days. The presence of an antheridiogen system was investigated using culture media extracts of *T. ovata* gametophytes. Young gametophytes of *T. ovata* and *Onoclea sensibilis* L. form antheridia prematurely when exposed to partially purified culture media extracts. Two bands of biological activity were present after thin-layer chromatography, indicating the presence of at least two biologically active compounds.

Studies of gametophyte growth and development play an important role in identifying new species of ferns with antheridiogen systems. Antheridiogens influence growth and development of gametophytes and their sexual morphology. They are synthesized by fern gametophytes and cause premature antheridia formation in developing gametophytes. Antheridiogen systems have been described in several genera of ferns, including *Anemia, Ceratopteris, Lygodium, Onoclea,* and *Pteridium* (reviews by Näf et al., 1975; Raghavan, 1989). In the schizaeaceous ferns, *Anemia* and *Lygodium,* antheridiogens also can substitute for the light requirement for spore germination. Morphological characteristics of fern gametophytes have been suggested to be of value in the classification of some fern groups (Stokey, 1951; Atkinson and Stokey, 1964). These morphological characteristics are obtained by studies involving growth and development of fern gametophytes from spore germination to reproductive maturity. Unfortunately, these studies are few considering the number of fern species (approximately 10,000 species; Bold et al., 1987). Differences between ferns concerning spore germination and prothallial development have been determined (Nayar and Kaur, 1971). The influence of environmental conditions such as light intensity and quality on gametophyte development in a limited number of species has also been investigated (review by Raghavan, 1989).

This study describes gametophyte development in *Thelypteris ovata* R.P. St. John var. *lindheimeri* (C. Chr.) A.R. Sm. from spore germination to mature sexual gametophytes at 48 days after spore sowing. Little detailed research has been done concerning gametophyte development in this species. *Thelypteris ovata* is native to Mexico, the Caribbean Islands, and the southeastern U.S. west to central and southwestern Texas. It often grows in wet soils along riv-

erbanks (Lellinger, 1985) and also is cultivated as an ornamental (personal observation). The var. *lindheimeri* is commonly called Lindheimer's maiden fern.

Partial purification of an antheridiogen in *T. ovata* var. *lindheimeri* was undertaken after preliminary studies in our laboratory indicated the presence of an antheridiogen system. The presence of an antheridiogen in *Thelypteris hexagonoptera* (Michx.) Weath. was demonstrated using *Onoclea sensibilis* L. as the bioassay organism, and *T. hexagonoptera* and *Thelypteris* sp. (Dryopteroids) have been shown to be sensitive to the antheridiogen of *Pteridium aquilinum* (L.) Kuhn (Näf, 1956); however, few details were provided in that study. The present study is the first to report an antheridiogen system in *T. ovata*. *Onoclea sensibilis* also is shown to be sensitive to the *T. ovata* antheridiogen.

Materials and Methods

Spores of *T. ovata* var. *lindheimeri* (referred to hereafter as *T. ovata*) were collected from D'Hanis, in Medina County Texas, USA, May, 1995, and stored in glass vials at 4°C until used. Good spore viability was obtained with this storage temperature. A voucher specimen collected by J. Nester-Hudson was deposited in the Warner Herbarium, Sam Houston State University, Huntsville, Texas.

Spores were surface sterilized with calcium hypochlorite (1%, w/v) using aseptic procedures as described previously (Schedlbauer, 1976), except that the overnight soak was omitted. Spores were then sown onto the surface of 0.6 % (w/v) agar-solidified mineral medium containing Parker's macronutrients and Thompson's micronutrient (Klekowski, 1969) with the addition of one drop of 1% ferrous chloride per liter instead of the Fe-EDTA. Gametophytes were grown in sterile plastic Petri plates (100 × 15 mm) under fluorescent light on a 16-hr light (approximately 150 lumens/ft_2), 8-hr dark cycle at 20–24°C.

For studies of growth and development, microscopic slides were prepared of 15–30 gametophytes every 2–3 days for 48 days. Gametophytes were fixed with Hoyer's mountant/fixative and stained with 0.5% acid fuchsin. Gametophytes on these slides were photographed using a Nikon photomicroscope with Kodak T-MAX 100 film. Gametophyte size was determined from prepared slides using an ocular micrometer and compound microscope. This study was replicated three times and similar results were obtained in each study.

In another study, individual gametophyte growth and development were monitored for 28 days to determine the time of spore germination, meristem formation and reproductive morphology at 28 days for at least 200 gametophytes. Spores were sterilized and sown as described above. In order to monitor germination and growth of individual gametophytes, a 2 cm^2 square of black plastic screening was taped to the underside of at least 5 plates and ungerminated spores in the screen grid were plotted on a separate sheet of paper. The screening facilitated the plotting of ungerminated spores and aided in monitoring individual gametophyte growth. Spores and subsequent game-

tophytes were observed daily for 28 days after spore sowing. After 28 days, most cultures were too crowded for further observations. This study also was repeated three times with similar results each time.

Scanning electron micrographs of spores were obtained with a JSM 330A scanning electron microscope. Spores were mounted onto double-stick tape, sputter coated with gold palladium, and viewed at 15 kV.

For studies of antheridiogen activity, spores of *T. ovata* were sown aseptically onto the surface of agar-solidified mineral medium in 100 × 15 mm plastic Petri plates and placed under fluorescent lights. Eighteen plates were staggered in three layers in aluminum pans, allowing a large number of plates with gametophytes to be grown in a limited space. No visual growth differences between the bottom and top layers could be observed. After two months, gametophyte culture media were frozen and then thawed to obtain an aqueous extract. This extract was adjusted to pH 2.5 with 2 M HCl and partitioned 3× with ½ volume of ethyl acetate. The ethyl acetate extract was dried with $MgSO_4$, filtered through Whatman No. 1 filter paper, and evaporated to near dryness. One liter media equivalents were placed in silylated glass vials (5% dimethydichlorosilane in toluene), evaporated to dryness and stored at -20°C. The residue (equivalent to one liter) was dissolved in ethanol and applied to a 20 mm^2 glass-backed thin-layer chromatography (TLC) plate (silica gel 60A, 0.25 mm thick, Whatman International Ltd. Maidstone, England) and chromatographed 15 cm in ethyl acetate:chloroform:acetic acid (75:25:5; v/v/v). Plates were divided into ten equal strips and compounds were eluted from the silica gel with methanol. These fractions were placed in silylated glass vials, evaporated, and stored at −20°C for subsequent analysis by bioassay.

For bioassays of antheridiogen activity involving the premature formation of antheridia by gametophytes of *T. ovata* and *O. sensibilis,* fractions after TLC were dissolved in ethanol and a portion equivalent to 300 ml original culture media was added to 15 ml liquid culture medium that was buffered with 25 mM MES (2-[N-Morpholino]ethanesulfonic acid) at pH 6.0. This bioassay media also was diluted 50%. *Onoclea sensibilis* spores were collected on the grounds of Sam Houston State University in Dec. 1995 and stored at -20°C until used. These spores were rinsed several times in sterile water before sowing and no sterilant was used. Liquid mineral media with a portion of TLC fractions were placed in 35 x 10 mm sterile plastic Petri plates in which a piece of Millipore filter (type HA, 0.045 mm) was placed on top of a ring of Tygon tubing (2 mm thick × 1.5 mm diameter). A sufficient quantity of liquid media (approx. 2 ml) was added to each plate to wet the filter paper. New spores of *T. ovata* and *O. sensibilis* were sown onto the surface of the filter paper in replicate cultures. This bioassay arrangement allowed gametophytes to grow on a firm surface, yet be assayed with the convenience of liquid culture media. These bioassays were placed under florescent lights. Microscope slides were made of gametophytes after 14 days for *O. sensibilis* and after 17 days for *T. ovata* for observations of the formation of premature antheridia. This experiment was repeated at least three times.

In a separate bioassay, *T. ovata* and *O. sensibilis* spores were sown on liquid

media with TLC extracts and placed in darkness to test for induction of dark germination of spores. Cultures placed in darkness were checked at 7 days for germination (since most germination had occurred in control cultures by 7 days) and then placed in the light for an additional 10 days for *O. sensibilis* and 14 days for *T. ovata* for observations of premature antheridia formation. This experiment was repeated three times.

Gametophytes of *T. ovata* also were bioassayed with gibberellic acid (GA_3). A dilution series of 1 mM, 0.1 mM, 0.01 mM, 0.001 mM, and 0.0001 mM GA_3 was bioassayed as described above and repeated three times. Bioassays were placed in the light and darkness. Microscopic observations of spore germination in darkness were made eight days after sowing. Microscope slides were made after 14 days for detection of antheridia formation in *T. ovata* of bioassays in the light.

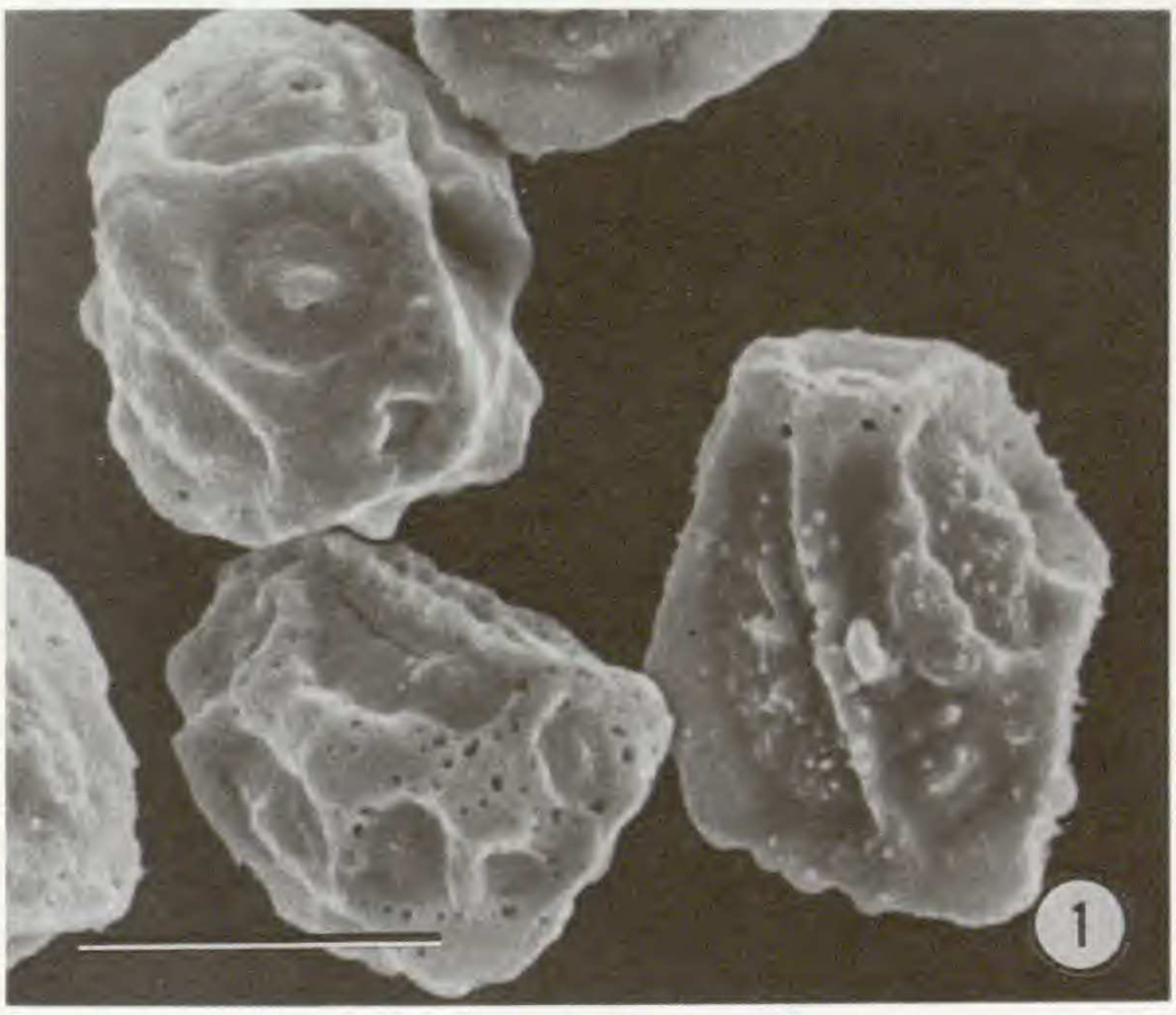
Results

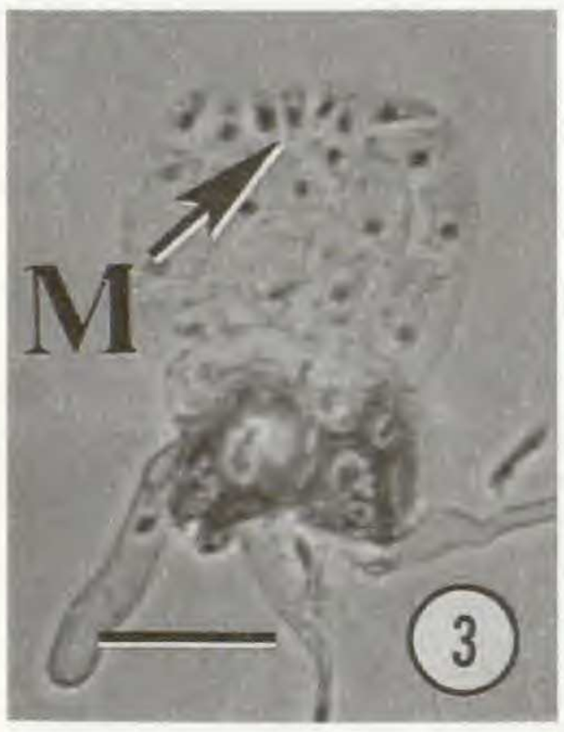
Spores of *T. ovata* are monolete, approximately 45 μm long × 30 μm wide, and have continuous connected folds that are 2–8 μm wide (Figs. 1, 2). The pattern of the folds varies, and folds may or may not have small perforations that vary in size. In mass, they are black in color.

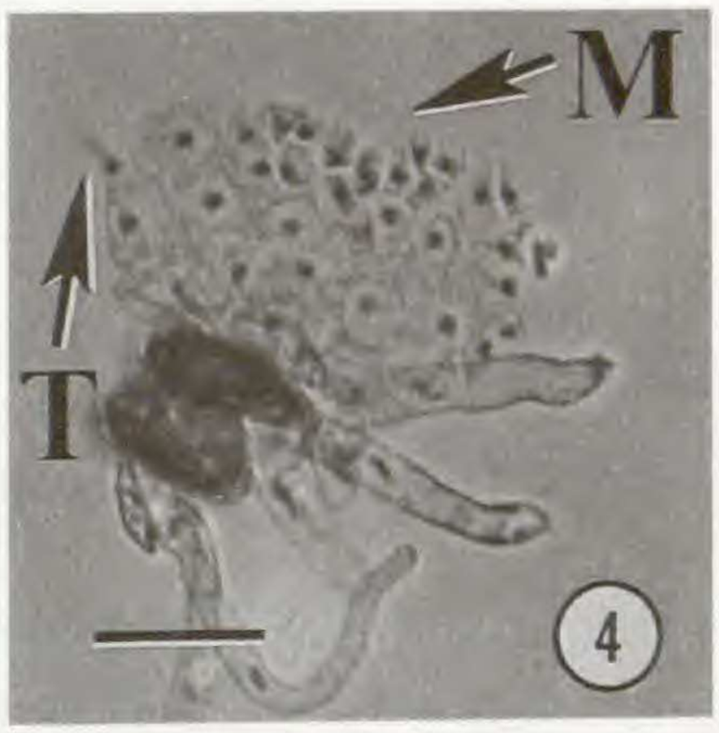
Spore germination began four days after sowing. Some spores germinated as late as nine days and among spores that were in cultures observed daily, none germinated after nine days. Approximately 90% of viable spores had germinated by day seven. A prothallial cell and a single rhizoid were observed emerging from the spore coat at germination. Some gametophytes were two dimensional at six days with two rhizoids formed while others had recently germinated. By day 10, prothalli were either spatulate (Fig. 3) or more fan-shaped (Fig. 4) with 2–5 rhizoids formed at the basal region. Initiation of a terminal organized meristem was evident, with cells of this meristem more thin and elongate when compared to other marginal cells. In some gametophytes, cells at the basal region were large and formed small lobes (Fig. 5). Unicellular marginal trichomes began to develop at this time, located on the outer margins of the prothallus (Fig. 4). From gametophytes observed daily, an organized meristem was initiated 5–9 days after germination in 75% of gametophytes.

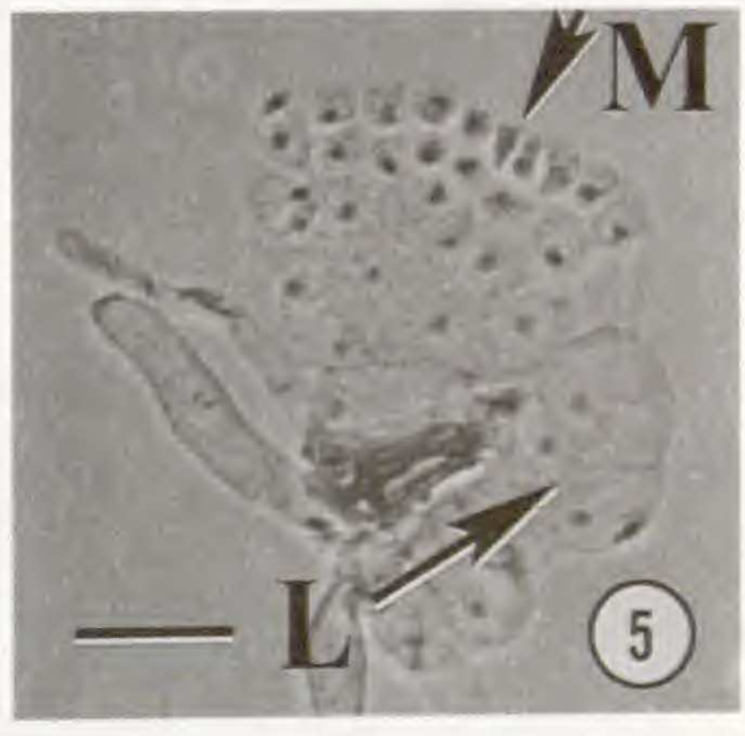
At 12 days, gametophytes varied in size, with some gametophytes considerably larger than others. Larger gametophytes measured 0.2–0.3 mm in width with prominent notched meristems and trichomes along the margin (Fig. 6). Trichomes were slightly swollen at the tip with prominently stained nuclei (Fig. 7). Smaller gametophytes were 0.1–0.2 mm in width. Some lacked an organized meristem, whereas others had an organized meristem, but lacked a notched meristem.

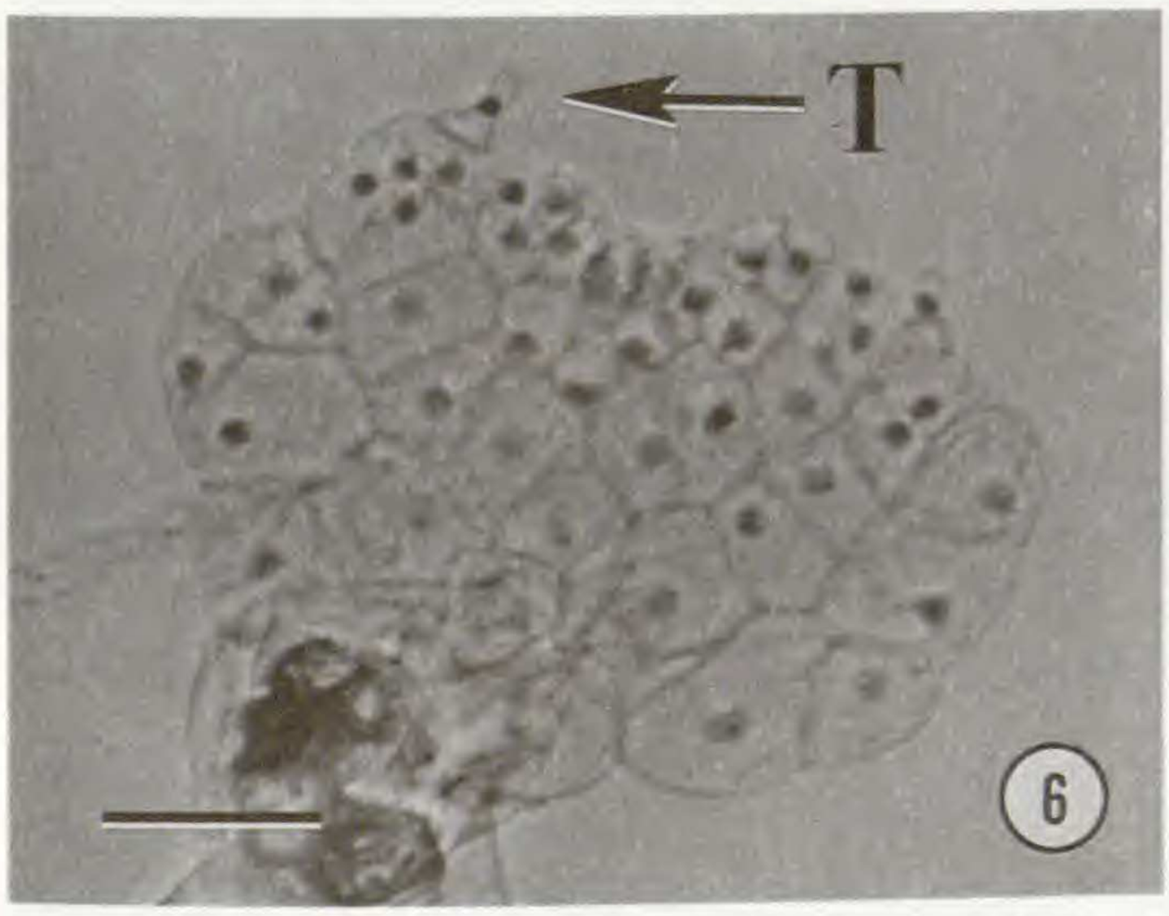
At 19 days a small percentage of gametophytes had begun to form antheridia while the majority of gametophytes remained asexual. Antheridia were formed on the surface of small (0.3–0.6 mm in width) cordate gametophytes near the

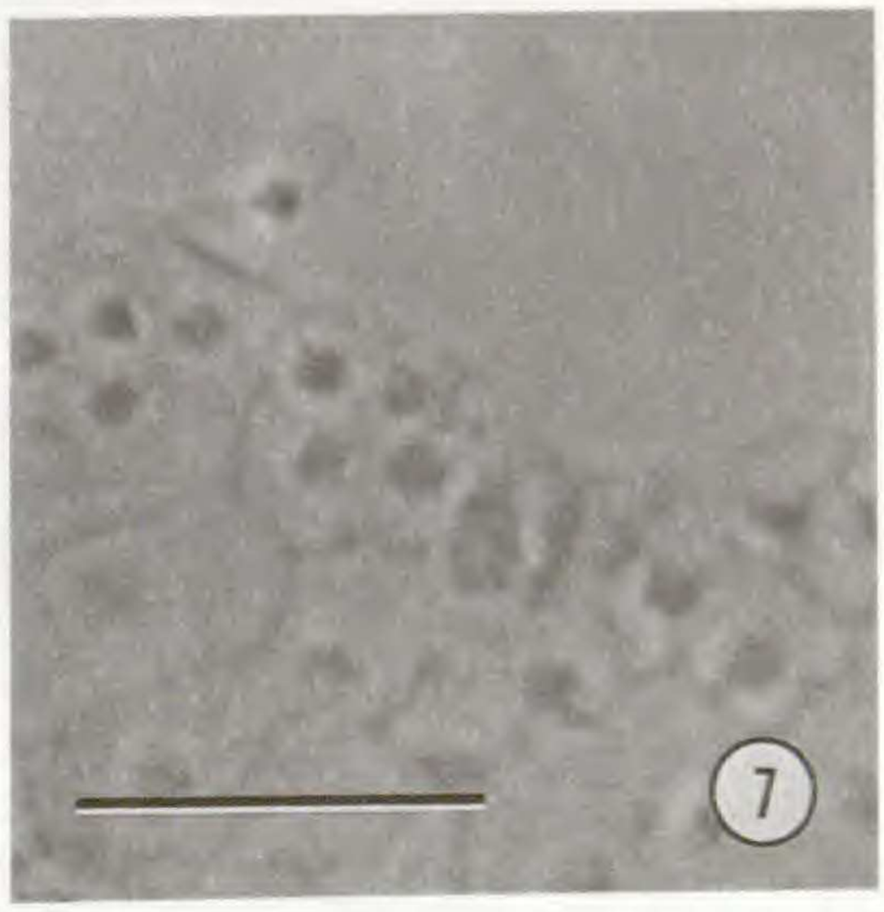












FIGS. 1–7. Spores and young gametophytes of *Thelypteris ovata* var. *lindheimeri*. Fig. 1. SEM of monolete spores with continuous folds. Bar = 25 μm. Fig. 2. SEM of spore with perforations. Bar = 10 μm. Fig. 3. Spatulate gametophyte with organized meristem (M) at 10 days. Bar = 50 μm. Fig. 4. Fan-shaped gametophyte at 10 days with organized meristem (M) and marginal trichome (T). Bar = 50 μm. Fig. 5. Gametophyte at 10 days with organized meristem (M) and basal lobe (L). Bar = 50 μm Fig. 6. Cordate gametophyte at 12 days with marginal trichome (T). Bar = 50 μm. Fig. 7. Enlargement of trichome with dark-staining nucleus from Fig. 6. Bar = 50 μm.

basal region (Fig. 8). Antheridia were spherical and measured 0.04 mm across (Fig. 9).

Young archegonia were distinguished as early as 21 days on gametophytes prepared for light microscopy (Figs. 10, 11). Archegonial necks were observed on living gametophytes at 24 days. Archegonia developed behind the notched meristem and archegonial necks curved away from the notched meristem. At 24 days, archegoniate gametophytes measured 0.9–1.4 mm in width. At this time, numerous multicellular trichomes could be seen developing on the surface of the gametophytes (Fig. 12) as well as along the margin.

At 28 days, gametophytes were antheridiate, archegoniate, or asexual. Antheridia continued to form on the surface of small (0.6–1.0 mm in width) cordate gametophytes (Fig. 13). As many as 20 antheridia formed on some antheridiate gametophytes, and antheridia varied in maturity from those with mature coiled sperm to those in which the spermatogenous tissue was undergoing mitosis (Fig. 14). Archegonia continued to form behind the notched meristem of large (1.3–2.3 mm in width) cordate gametophytes. One to five archegonia formed per gametophyte. Asexual gametophytes varied in size from 0.5 to 1.5 mm in width.

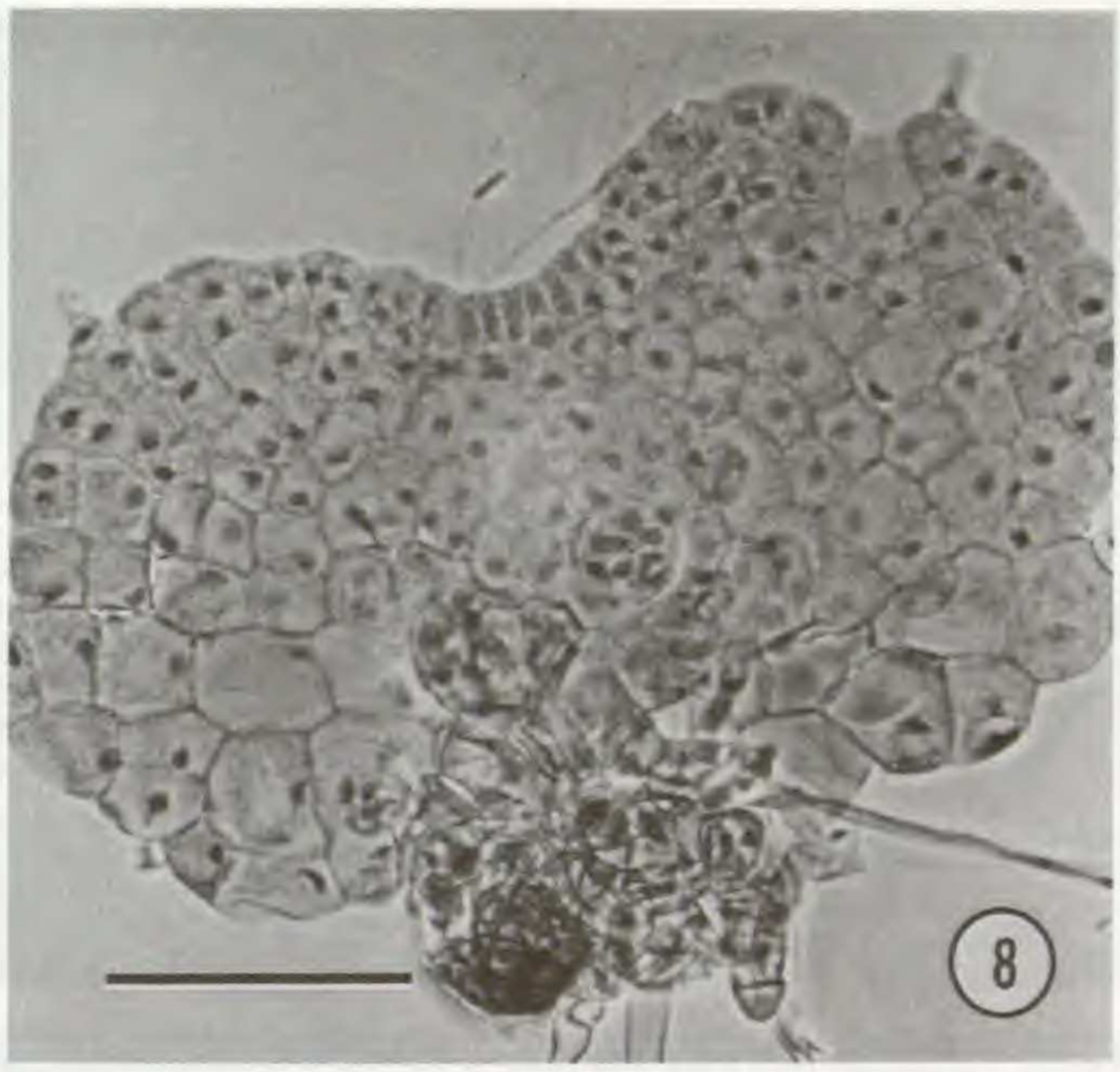
At 48 days, almost all gametophytes were cordate. Approximately 10% of gametophytes were antheridiate while most were archegoniate, and less than 10% remained asexual. No bisexual gametophytes were observed. Throughout this study, archegoniate gametophytes were larger than antheridiate gametophytes and cordate asexual gametophytes were variable in size.

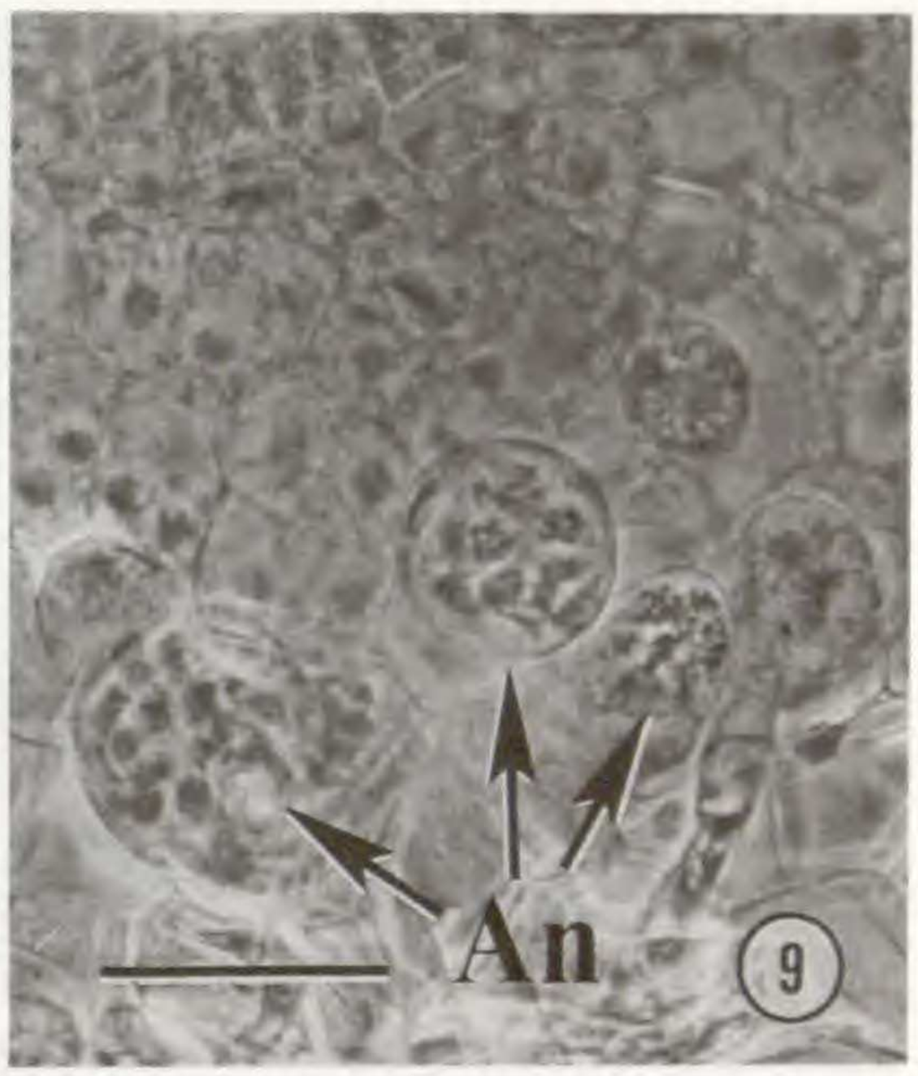
Gametophytes that were not crowded in the Petri plates were flat and cordate with very little ruffling of the wings. Those growing in crowded conditions were often growing in a more upright position. Wings remained one-cell thick with the midrib several cells thick.

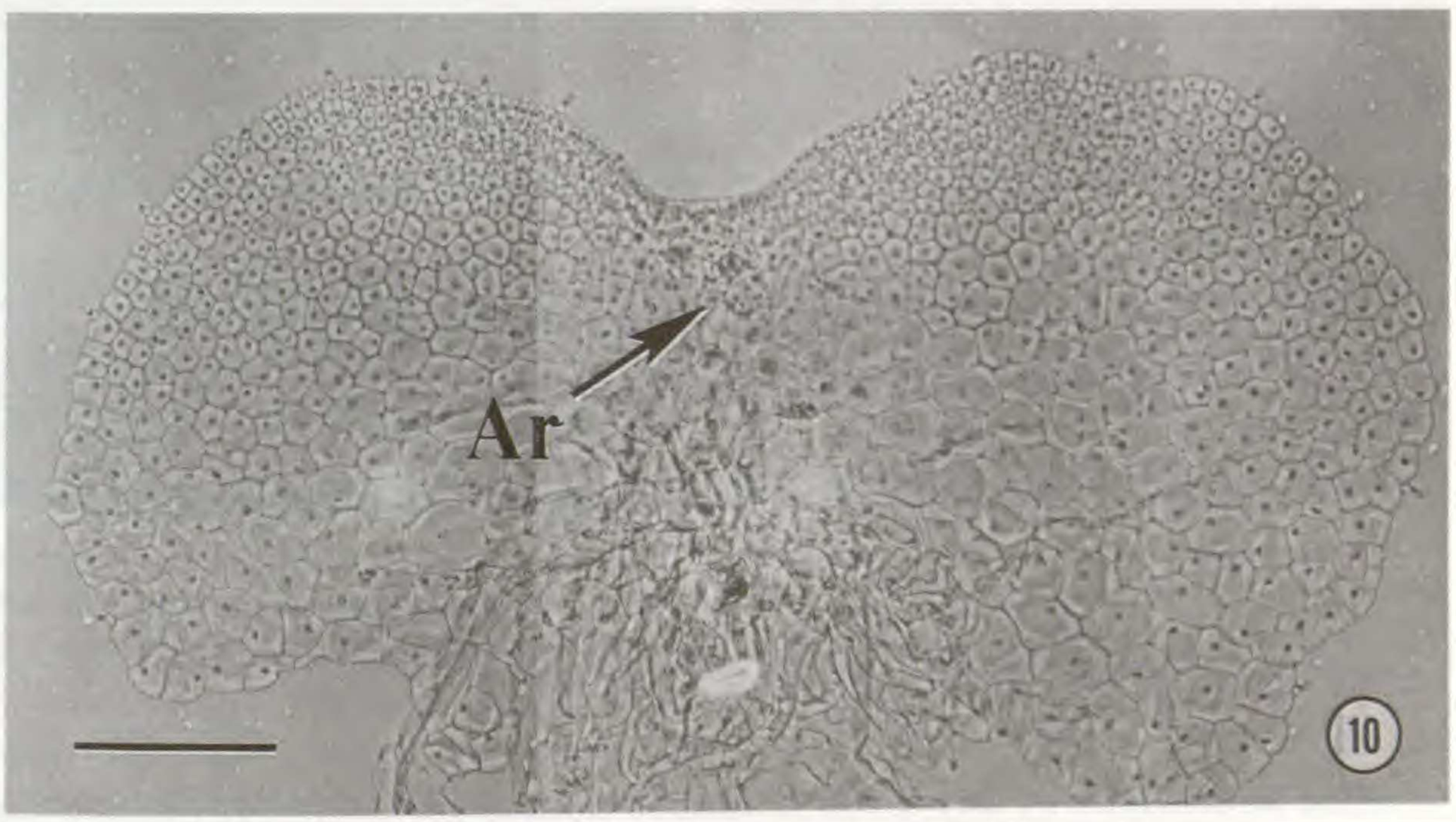
Observations of gametophytes studied daily suggested that time of spore germination or time of initiation of organized meristem had no influence upon whether the gametophyte was antheridiate, archegoniate or asexual at 28 days. Crowding of gametophytes did not appear to influence the number of antheridiate gametophytes.

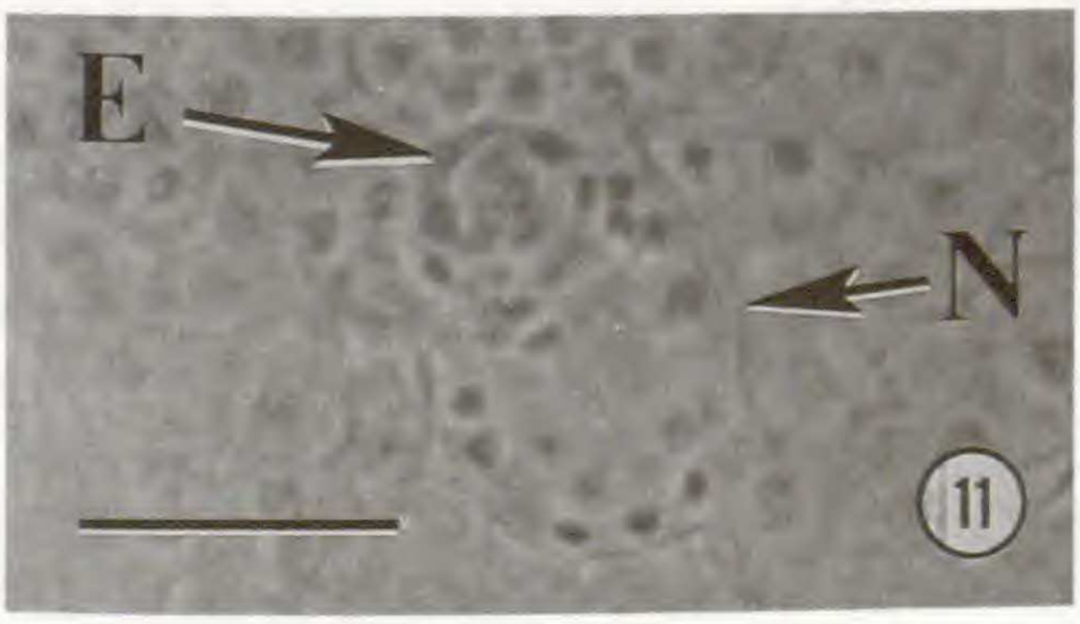
The presence of an antheridiogen system in *T. ovata* had been suggested from previous preliminary studies in our laboratory. Further work involved partial purification of an antheridiogen with TLC. Gametophytes of *T. ovata* and *O. sensibilis* form multiple antheridia prematurely in response to antheridiogen synthesized by *T. ovata* gametophytes (Figs. 15,16). The acidified ethyl acetate extract of culture media after fractionation by TLC showed two bands of biological activity at R_f 0.3–0.4 and 0.8 (Fig. 17). Similar results were obtained with bioassays of *O. sensibilis* gametophytes. Spore germination for both species was greater than 90% in these bioassays.

Spores of *T. ovata* and *O. sensibilis* do not germinate in darkness in response to antheridiogen. In light grown cultures, approximately 90% of spores had germinated by day seven. When these ungerminated spores were subsequently placed in the light and allowed to germinate and grow for 10–14 days, antheridia were initiated. In response to GA_3, no gametophytes of *T. ovata* ger-

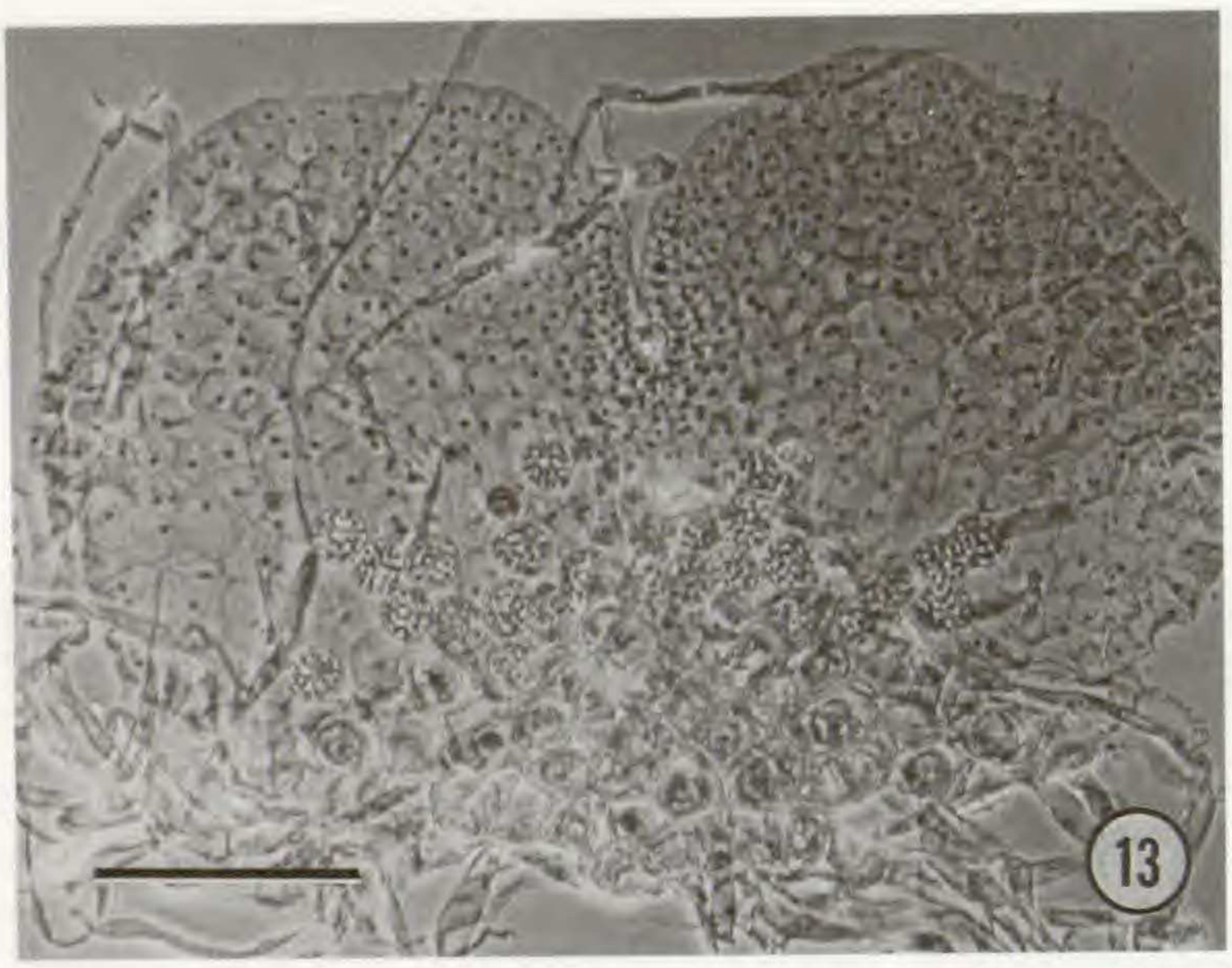


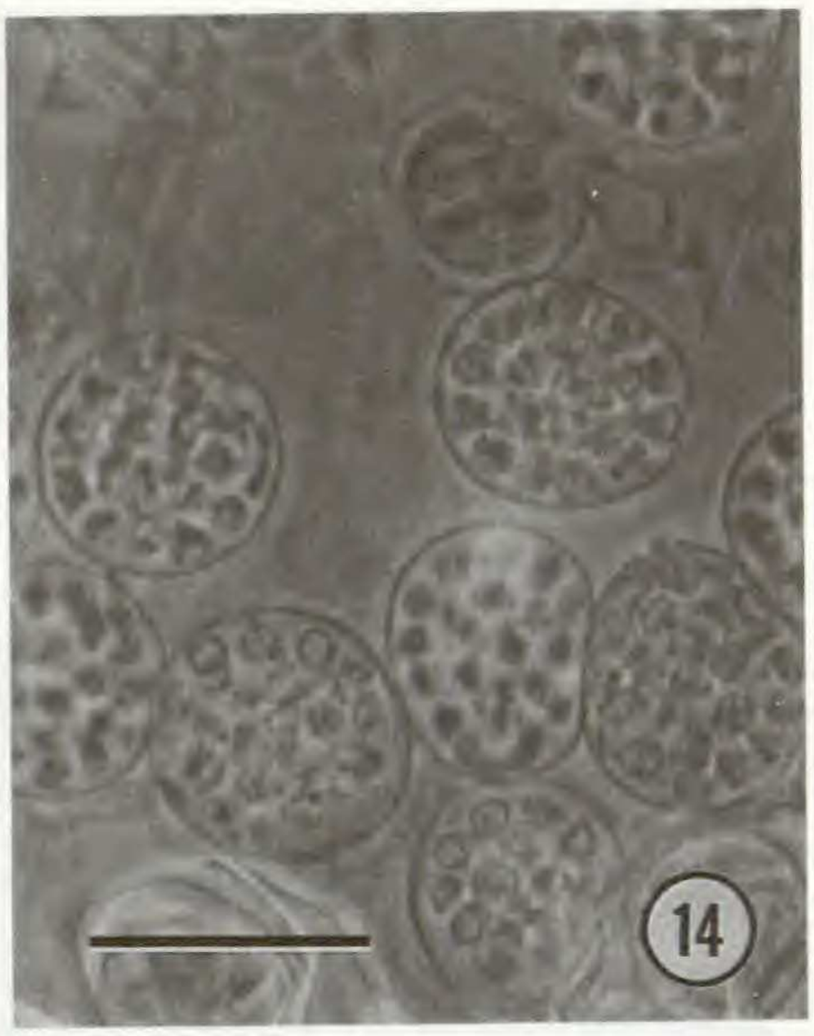


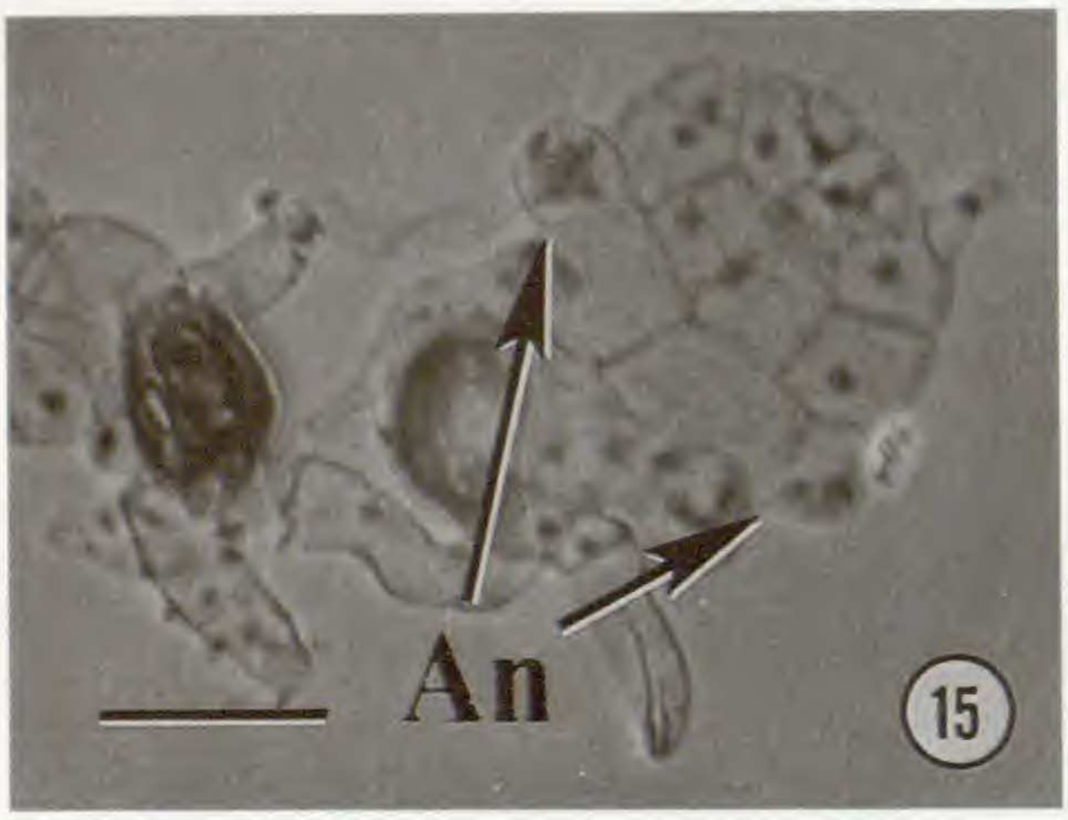


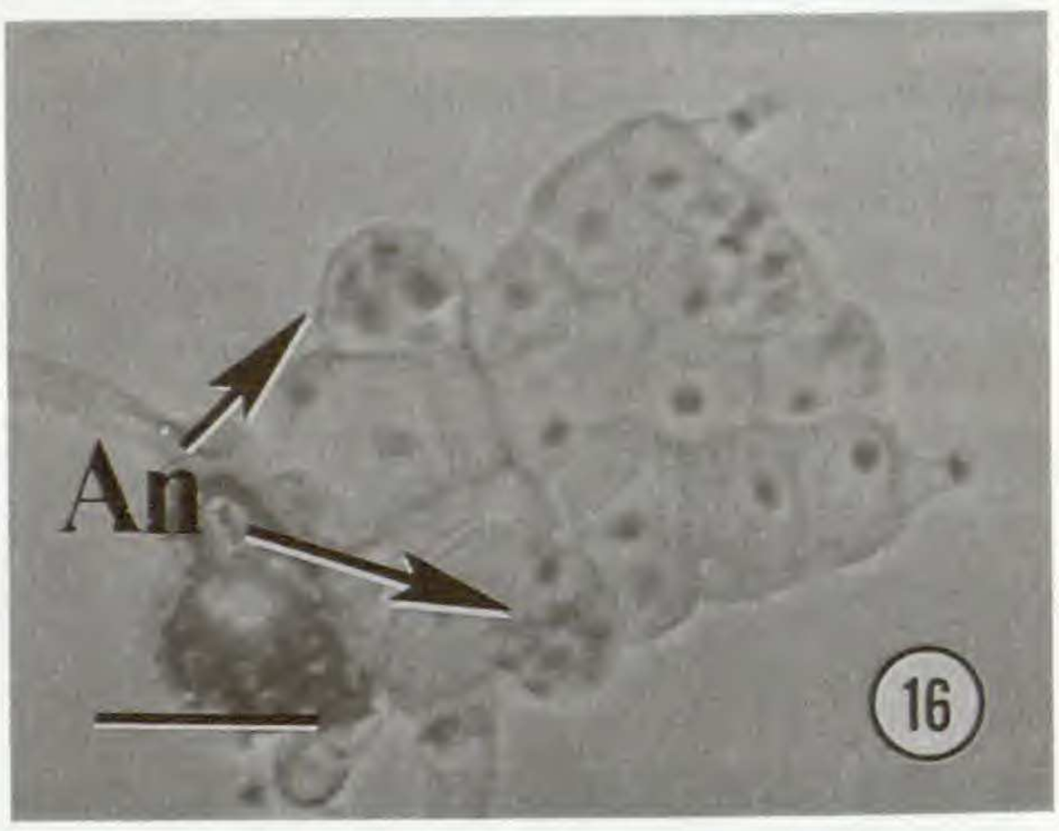


FIGS. 8–12. Gametophytes of *Thelypteris ovata* var. *lindheimeri*. Fig. 8. Cordate gametophyte with antheridia at 20 days. Bar = 100 μm. Fig. 9. Enlargement of antheridia (An) from Fig. 8. Bar = 50 μm. Fig. 10. Cordate gametophyte with archegonium (Ar) at 24 days. Bar = 200 μm. Fig. 11. Enlargement of archegonium from Fig. 10 with archegonial neck (N) and egg (E). Bar = 50 μm. Fig. 12. Surface trichomes (T). Bar. = 50 μm.









FIGS. 13–16. Gametophytes of *Thelypteris ovata* var. *lindheimeri*. Fig. 13. Antheridiate gametophyte at 28 days. Bar = 200 μm. Fig. 14. Enlargement of antheridia from Fig. 13. Bar = 50 μm. Figs. 15, 16. Fourteen day old gametophytes which have formed antheridia (An) prematurely in response to antheridiogen partially purified by TLC. Bar = 50 μm.

minated in darkness by day seven or formed antheridia in the light at any GA_3 concentration used in this study.

DISCUSSION

Spores of *T. ovata* var. *lindheimeri* are monolete with distinct continuous folds. This spore morphology is similar to *T. ovata* var. *ovata, T. flaccida* (Blume) Ching, and *T. reptans* (J. F. Gmel.) C. V. Morton (Tryon and Lugardon, 1991). This general morphology also has been described for *T. cordata* (Fée) Proctor and *T. brunnea* (C. Chr.) Ching (Wood, 1973). Some *Thelypteris* species have spores with short spines on the surface (Wood, 1973; Tryon and Lugardon, 1991). These spines are not present on *T. ovata* var. *lindheimeri* spores.

Spore germination occurred 4–9 days after spore sowing. In other *Thelypteris* species, this also has been observed (Kachroo, 1963; Smith, 1971). The pattern of cell divisions at germination has been studied by Huckaby and Rag-

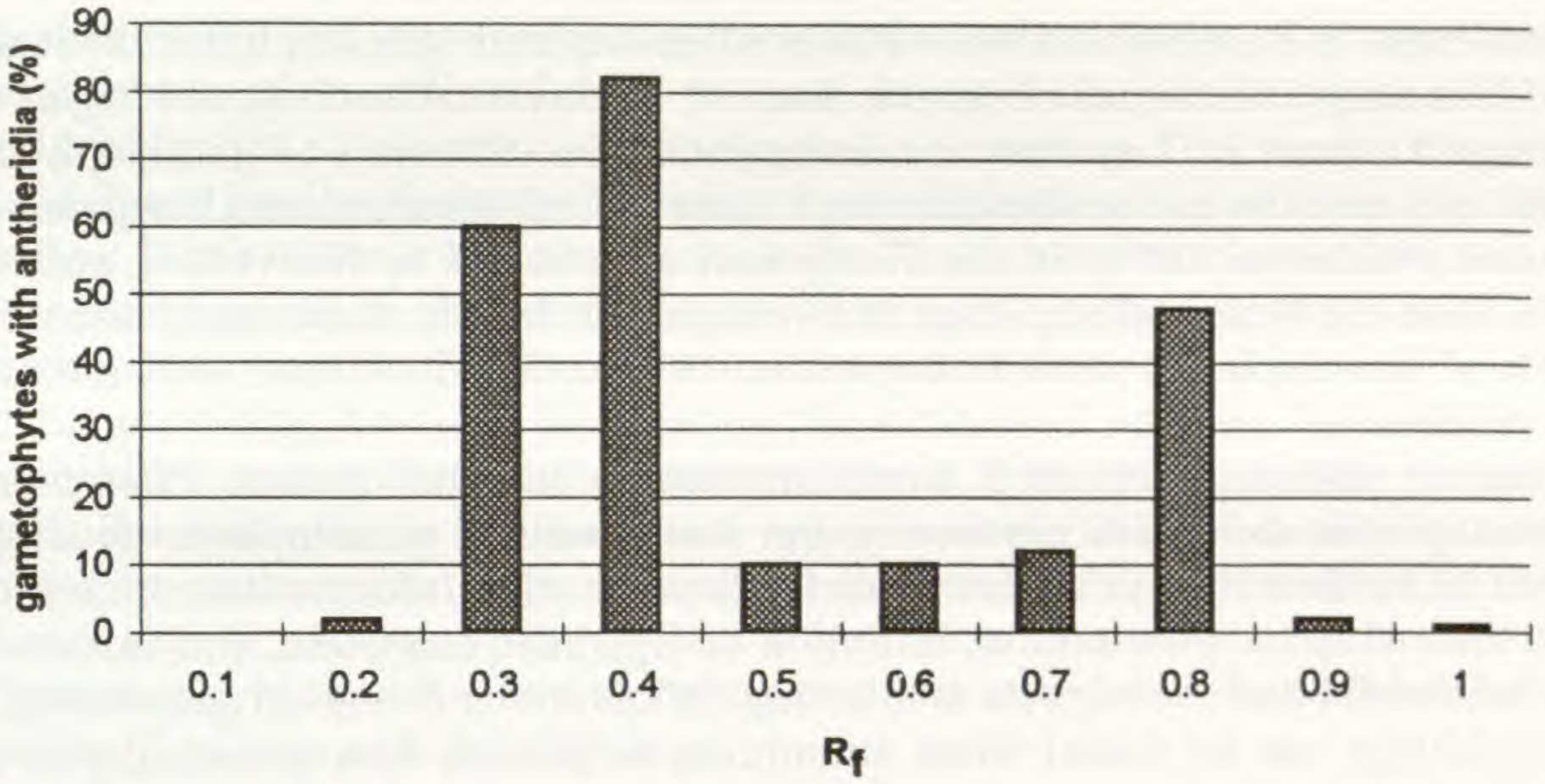


FIG. 17. Percentage of *T. ovata* gametophytes with antheridia in response to gametophyte culture media extract of *T. ovata* after fractionation by TLC (ethyl acetate:chloroform:acetic acid, 75:25:5).

havan (1981) for several *Thelypteris* species using both sectioned material for light microscopy and intact material for SEM. In their work, it was found that the spore coat cracked open along the monolete mark and the first asymmetric division of the spore cell resulted in a rhizoid cell and basal cell. The basal cell divided again to form a protonemal cell. In the present study, the cell division pattern at germination could not be determined because sectioned material was not studied.

Gametophytes of *T. ovata* did not form a noticeable filament and were two-dimensional almost immediately. This differs from most other *Thelypteris* species that have been studied. In *Cyclosorus molliusculus* (Kuhn) Ching, spores germinated seven days after sowing and gametophytes were initially a three-celled filament (Kachroo, 1963). In several *Thelypteris* species, a three to six-celled filament formed (Nayar and Chandra, 1963; Atkinson, 1971, 1975; Atkinson and Stokey, 1973).

At 12 days, gametophytes of *T. ovata* have well-developed organized meristems and several unicellular marginal trichomes (Fig. 6). Gametophyte development in *T. ovata* follows the *Drynaria* type described by Nayar and Kaur (1971). Unicellular marginal and surface trichomes have been described in other *Thelypteris* species, although time of initiation of trichomes varied between species (Kachroo, 1963; Nayar and Chandra, 1963, 1965; Atkinson, 1971, 1975; Smith, 1971; Atkinson and Stokey, 1973). In *T. erubescens* (Wall. ex Hook.) Ching, some gametophytes have marginal trichomes at one month, but others lack trichomes (Atkinson, 1971). No glandular secretions were observed. Acicular trichomes have been reported in some *Thelypteris* species (Atkinson and Stokey, 1964).

In *T. ovata,* antheridia and archegonia are on separate male and female gametophytes when gametophytes are grown in multispore cultures. Archegoniate gametophytes are considerable larger than antheridiate gametophytes.

Antheridia and archegonia were observed on separate gametophytes throughout this study. Antheridia began to form at 19 days and archegonia began to form at 21 days. In *T. erubescens,* archegonia were observed on gametophytes after two months and antheridia were observed on separate, smaller gametophytes (Atkinson, 1971). In the *Thelypteris* sp. studied by Näf (1956), antheridia were not formed at any stage of development. In this study, approximately 80% of gametophytes were archegoniate, 10% antheridiate, and 10% asexual at 48 days.

Mature gametophytes of *T. ovata* are similar to other mature *Thelypteris* gametophytes described previously, but few details of gametophyte development in the first 30 days are available for these species. Information concerning the time of spore germination, initiation of organized meristem, and initiation of antheridia and archegonia is limiting. Trichome and overall gametophyte morphology can be useful when attempting to identify fern gametophytes in nature (personal observation), and Atkinson and Stokey (1964) presented evidence that fern gametophytes could be used taxonomically. Differences between gametophytes of various species can only be determined through detailed studies of growth, development, and morphology.

The presence of antheridiate and archegoniate gametophytes at the end of 48 days suggested that an antheridiogen system may be present in this species. Preliminary studies in our laboratory verified the presence of an antheridiogen system and partial purification of the antheridiogen was undertaken. The results of bioassays with *T. ovata* and *O. sensibilis* indicate the presence of more than one biologically active compound. This is not unusual as two or more antheridiogens have been detected and/or chemically characterized in *Anemia phyllitidis* (L.) Sw. (Oyama, et al., 1996), *A. mexicana* Klotzsch (Nester et al., 1987), *Lygodium japonicum* (Murr.) Sw. (Yamane et al., 1979, 1988), *L. circinnatum* (Burm. f.) Sw., and *L. flexuosum* (L.) Sw. (Yamauchi, et al., 1996). The biologically active compounds of *T. ovata* will be purified further by ion-exchange chromatography and high performance liquid chromatography for eventual chemical characterization.

Spores of *T. ovata* and *O. sensibilis* are not induced to germinate in darkness in response to antheridiogen of *T. ovata.* These results are as expected when compared to results of antheridiogen studies in most other non-schizaeaceous ferns. However, Chiou and Farrar (1997) recently demonstrated that antheridiogens in the Polypodiaceae can induce spore germination in darkness after 30 days. Since greater than 90% of *T. ovata* spores germinate in the light by day seven, bioassays were observed at day seven.

Spores of *T. ovata* are not induced to germinate in darkness in response to GA_3. These results also are expected when compared to results of GA_3 activity in most other non-schizaeaceous ferns.

Antheridiogen systems have been described in other fern species (review by Näf et al., 1975; Raghavan, 1989). *Thelypteris hexagonoptera* and *Thelypteris* sp. have been shown to be sensitive to the antheridiogen of *P. aquilinum,* and the presence of an antheridiogen in *T. hexagonoptera* was demonstrated using *O. sensibilis* as the bioassay organism (Näf, 1956). Although the *Thelypteris*

sp. studied by Näf (1956) was sensitive to the antheridiogen of *P. aquilinum,* the presence of an antheridiogen was not demonstrated in that species. This study is the first to report an antheridiogen in *T. ovata* and the second detected in the large family Thelypteridaceae, of which there are approximately 1000 species. Further work will require biological and chemical characterization of these compounds. In the search for new ferns with antheridiogens, studies of gametophyte growth, development and reproductive morphology will be useful for providing baseling information for individual species. Percentages of antheridiate and archegoniate gametophytes at maturity may differ between ferns. For example, in *A. mexicana,* approximately 80% of cordate gametophytes are antheridiate, 10% are archegoniate and 10% are asexual at 60 days (Nester and Schedlbauer, 1981). This information is essential for discussions of gametophyte reproductive strategies and can only be obtained by detailed studies of growth and development.

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